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(57) Abstract

One or more pure cultures of *Lactobacillus*, such as *L.reuteri*, *L.animalis* and *L.salivarius* and a sugar source, such as whey and a method of feeding animals which utilizes the formulation to be ingested by the animals with their normal food. Preferably, direct feed microorganisms such as *Lactobacillus reuteri* are established in the gastrointestinal tract of avian organisms, adding them to whey and feeding the composition in the form of pellets to the organisms.

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FEED ADDITIVE AND METHOD

FIELD OF INVENTION

This invention relates to a new method for delivering viable microbial cells in animals' diets and for reducing microbial pathogens such as Salmonella.

BACKGROUND INFORMATION

Under certain conditions some members of the indigenous gastrointestinal microbiota can become opportunistic pathogens causing a variety of enteric diseases. More often, however, pathogens gain access to the GI tract as contaminants in food or water. Notable among the latter are a number of bacterial genera including Escherichia, Salmonella, Shigella, Yersina, Vibrio, Campylobacter and Clostridium, as well as viruses (e.g., roto-, astro- and ciliciviruses) and intestinal parasites (e.g., Giardia and Entamoeba species). Acute and chronic enteric diseases caused by these and other microorganisms occur worldwide causing considerable human misery and loss of economically important animals. Certain microbial activities have also been associated with production of mutagens within the GI tract.

It is known that other members of the indigenous microbiota exist in a symbiotic or synergistic relationship with their host contributing in many positive ways to the host's general health and well-being. It is well-known that germ-free animals are very susceptible to pathogens and have poorly developed GI tracts. In return for the nutrient-rich and stable ecosystem provided for them, the indigenous microbiota can provide their hosts with an assortment of benefits including among others protection against enteric pathogens (a process known as colonization resistance or competitive exclusion), stimulation of normal development and function of the GI mucosa, production of various vitamins and other nutrients, and re-metabolism of

the host's abundant endogenous mucosal tissue.

It has been reported on numerous occasions that the enteric lactobacilli (i.e., bacteria belonging to the genus Lactobacillus which reside in the GI tract and which include a large number of nonpathogenic, non-toxic bacteria) play an important role in the health and well-being of their human and animal hosts.

The metabolic endproducts of Lactobacillus metabolism, such as acetic acid, lactic acid and hydrogen peroxide, are well-known for their antimicrobial activities. They are believed to play a significant role in maintaining proper conditions within the GI tract. Some lactobacilli produce bacteriocins or bacteriocin-like proteins which also exhibit bacteriocidal activity toward other members of that species or closely related species. Reports have appeared concerning low molecular weight, antimicrobial substances produced by lactobacilli. With the exception of reuterin which is produced by Lactobacillus reuteri, none of these low molecular weight substances has been identified and these reports have not been confirmed. In fact, some of these substances have proven to be none other than lactic acid, acetic acid or hydrogen peroxide.

Some of these beneficial microorganisms have been used as probiotics. The term "probiotics" is attributed to Parker (32) who defined probiotics as "organisms and substances which contribute to intestinal balance" when used as dietary supplements. This publication and all other publications and patents cited herein are incorporated herein by reference. Later, Fuller (17) considered this definition to be too broad since, in addition to including cell cultures and microbial metabolites, it could encompass antibiotic preparations. More recently, a number of summaries have appeared in the literature describing the scientific basis for use of probiotics as intestinal inoculants for production animals (15, 40). It has been suggested that the term "probiotics"

be replaced by the term "direct feed microorganisms," or DFM's (14).

It is generally held that during periods of low resistance, such as stress, undesirable microorganisms are able to proliferate in the GI tract of animals, humans included. Maintaining a normal, healthy balance of microorganisms is deemed to be critical during such stressful periods (15). The concept underlying use of DFM's, therefore is that if sufficient numbers of an appropriate microorganism(s) are introduced into the intestinal tract (i) at times of stress and/or disease, (ii) at birth, or (iii) after antibiotic treatment (when minimal LAB are present), the negative consequences of the microbial imbalances can be minimized or overcome. Using such preparations of live, naturally occurring microorganisms helps restore and maintain the proper balance of beneficial microbes in the GI tract during times of stress, disease, and following antibiotic therapy (15). This concept, descriptions of proposed modes of action, and evidence for the efficacious uses of DFM's for all production animals are summarized in reviews by Fox (15), Sissons (40), and by various authors (36).

The concept of adding viable, harmless lactic acid bacteria to the gastrointestinal tract as a dietary supplement was first appreciated by Metchnikoff (26) who viewed the consumption of yoghurt by Bulgarian peasants as conferring a long span of life. Some workers have claimed that the therapeutic value derived from ingestion of such fermented milk products is related to the viable bacteria present in these products (18, 42). Since Metchnikoff's early reports, several studies have shown the ability of lactobacilli, for example, to suppress coliform growth. Feeding viable Lactobacillus acidophilus cells to young dairy calves was shown to reduce the incidence of diarrhoea (4), and increase the numbers of lactobacilli and reduce coliform counts in feces (5). These findings contrast with

those of others who have been unable to demonstrate benefits from feeding either Lactobacillus acidophilus (13, 21) or milk cultured with Lactobacillus acidophilus or Lactobacillus lactis (27).

In a detailed study by Muralidhara et.al. (28), piglets given a Lactobacillus lactis concentrate for up to 8 weeks after birth showed a progressive decline in coliform counts in fecal samples. Scouring in these animals was negligible, but was evident in control pigs especially at weaning. Underdahl et al. (49) observed only mild diarrhoea lasting 2-4 days in gnotobiotic pigs inoculated with Streptococcus faecium prior to artificial Escherichia coli infection. In the same study, persistent diarrhoea occurred in pigs similarly infected with Escherichia coli, but without prophylactic treatment with the Streptococcus microorganism.

The lactic acid bacteria (LAB), particularly those classified in the following genera, are often used in probiotics: Lactobacillus, Lactococcus, and Enterococcus. Included among these are the following species: Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus plantarum, Lactobacillus casei, Lactobacillus lactis, Lactococcus lactis, Lactococcus thermophilus, Lactococcus diacetylactis, and Enterococcus faecium. Besides these LAB, some species of Bacillus (Bacillus subtilis, Bacillus toyoi) and yeasts and molds (Saccharomyces cerevisiae, Aspergillus oryzae, and Torulopsis sp.) are used as DFM's (15).

Certain Lactobacillus species in fact are added to human and animal foodstuffs either to preserve them, enhance their flavors and/or exert other beneficial effects in the GI tract. Lactobacillus plantarum strains, for example, are grown commercially in large amounts and used as starter cultures for the commercial preservation of a variety of human foods (meats, vegetables, and dairy products) and animal foods (silage).

Lactobacillus acidophilus strains are grown commercially in large amounts to be added to human (e.g., milk) or animal (feedstuffs) foods as a means of introducing these bacteria into the GI tract where they can exert beneficial effects. Although these bacteria are likely to be already present in the GI tract their numbers may vary widely from individual to individual, and therefore beneficial effects of these bacteria may not be present in persons deficient in these bacteria. Reports on the beneficial effects resulting from the oral administration of live Lactobacillus cultures have increased in recent years with findings that dietary Lactobacillus therapy affords protection from colon cancer for human populations on western diets, reduces the incidence of experimentally induced large bowel tumors in rats, reduces the fecal concentration of bacterial enzymes known to catalyze the conversion of procarcinogens to proximal carcinogens in humans, and reduces the serum cholesterol levels in swine.

Several studies have been conducted to determine the effect of lactobacilli on the performance of domestic avian species. Some of these studies indicate that dosing broilers with L. acidophilus improved their growth (11, 48). An increase in egg production as a result of addition of lactobacilli to laying hens feed has also been reported (20).

Improvement of young turkeys' body weight and feed efficiency was obtained with a Lactobacillus product added to the feed (16). Similar effects were reported (34) when L. acidophilus in combination with varying protein levels was fed to turkeys up to 12 weeks of age, but no difference with the controls was observed at 16 weeks. Dosage of 10^7 colony forming units (CFU) and higher depressed chick growth (51, 52).

Other strains of lactobacilli did not stimulate broilers weight gain (52), and did not stimulate egg

production (19). Damron et al. (9) did not find any beneficial effect of L. acidophilus and other lactobacilli cultures in turkey breeder hens.

Nurmi and Rantala (29) demonstrated that the intestinal microflora present in some adult chickens (i.e. the cecal microflora) interferes with colonization by salmonellae of newly hatched chicks. The application of this concept, known as the Nurmi concept or competitive exclusion, has been successfully tested in some laboratories and is also used commercially (22, 41, 53, 54). There are many problems associated with this method, in particular, a lack of adequate selective isolation and characterization techniques to study and consistently obtain cecal flora preparations (3, 25, 42).

Mannose and lactose were shown to significantly reduce Salmonella typhimurium adherence to the ceca of chicks (31). The inhibitory effect of these sugars was believed to take place by blocking the receptor sites on the gut epithelium and on the microorganism pili. It has been shown that providing dietary lactose together with cecal flora contents to broiler chickens reduced the occurrence of Salmonella (7, 8, 31).

Historically, Lactobacillus administration (i.e., inclusion of viable cells in the feed) to animals has not yielded consistent benefits. There are many reasons for this including, for example, using Lactobacillus species or strains unadapted to or unsuitable for the animal being treated, or using conditions which do not produce a colonization of the Lactobacillus within the GI tract.

One of the major problems or limitations encountered in commercial scale application of DFM's to animals is (i) the availability of suitable delivery systems, and (ii) the ability to get the probiotic preparations to the animals as quickly as possible after birth. This is particularly true when pelletized feeds are used, as is the case in the poultry industry. The pelletization process generally

includes one or more heating steps involving temperatures high enough to pasteurize or sterilize the feed components, thereby precluding incorporation of viable microorganisms into these feeds prior to pelletization.

The present invention describes novel methods and processes for overcoming some of these problems, by delivering viable DFM's in feed additives. Lactobacillus reuteri, along with L. animalis, and L. salivarius, which may be used in the invention, are naturally occurring microorganisms in the GI tract of animals including domestic avian species (38). The DFM used to develop these methods using pellets is Lactobacillus reuteri. This species was chosen because it has demonstrated efficacy as a DFM in poultry (33). This efficacy is also discussed in PCT/US88/01423, filed April 28, 1988 and published November 3, 1988, the disclosure of which is incorporated herein by reference.

Lactobacillus reuteri is a species of lactic acid bacteria recognized since the turn of the century (30). Originally assigned different species names (e.g., Lactobacillus fermentum biotype II), it obtained distinct species status in 1980 and is registered in the 1988 edition of Bergey's manual (23, 24). It is found in foods, particularly dairy products and meats, but exists primarily in the GI tract of healthy animals, including humans (1, 10, 12, 23, 24, 37, 38, 39, 50).

Lactobacillus reuteri is the dominant heterofermentative Lactobacillus inhabiting the GI tract (37, 38, 39). Lactobacillus reuteri is a symbiotic resident of the gastrointestinal (GI) tracts of humans, swine and other animals. The neotype strain of L. reuteri is DSM 20016 (ATCC No. 53609). This strain and strain 1063 (ATCC No. 53608), discussed in the co-pending application, are available to the public at the American Type Culture Collection (Rockville, MD) having been deposited therein April 17, 1987.

Lactobacillus reuteri is a typical heterofermenter, converting sugars into acetic acid, ethanol, and CO_2 in addition to lactic acid which is the major endproduct of homofermentative metabolism carried out by species such as Lactobacillus acidophilus (21). It utilizes the phosphoketolase pathway for conversion of glucose to endproducts. When glycerol, an alternate hydrogen acceptor, is present in the culture medium together with glucose or other utilizable carbon and energy sources (e.g., lactose), acetate rather than ethanol accumulates, and the glycerol is reduced to 1,3-propanediol via the metabolic intermediate, 3-hydroxypropionaldehyde (3-HPA). 3-HPA has been shown to have potent antimicrobial activity, and Lactobacillus reuteri appears to be unique among microorganisms examined to date in its ability to secrete this substance, termed reuterin, into the surrounding medium (2, 6, 12, 44, 45, 46, 47). This unique antimicrobial activity may play a role in competitive survival of this species in the gastrointestinal ecosystem, and/or its ability to regulate growth and activities of other microorganisms in this ecosystem (12). It is thus very important to establish this microorganism early in animals. It is therefore an object of the invention to provide a method for delivering DFM's, such as Lactobacillus, to avian species.

It is another object of this invention to provide a food or feed additive formulation and method comprising isolated and identified pure cultures of Lactobacillus reuteri and/or other Lactobacillus species together with a sugar source such as lactose, using whey as a source for this sugar.

It is a further object of the invention to provide a formulation that results in rapid weight gain for growing animals.

It is a further object of the invention to provide a formulation that decreases the number of pathogenic

microorganisms in the gastrointestinal tract, with the purpose of adding any sugar for at least the purpose of being a source of carbohydrate for the metabolism of the Lactobacillus but not utilized by the animal or the unwanted microorganism(s).

Other objects and advantages will be more fully apparent from the following disclosure and appended claims.

SUMMARY OF INVENTION

The invention includes a formulated product that may be used as an animal feed additive and that includes isolated and identified pure culture(s) of naturally occurring gastrointestinal microorganisms, for example, Lactobacillus reuteri, L. animalis, and/or L. salivarius, and a sugar source. The invention also includes a method of feeding the formulation to animals. Preferably the sugar source is whey, when animals which do not metabolize lactose such as chickens are used, because whey contains the sugar lactose and is an easily obtainable and voluminous waste product.

A dietary supplement is prepared containing viable cells of a DFM such as Lactobacillus reuteri, an oil and a cryoprotectant such as whey powder. The Lactobacillus cells may be coated on the surface of whey pellets or be contained in the pellets. As used herein, the word "pellet" means a compacted whey particle which may be of any size or shape that is ingestible by the animal to be fed the supplement.

The formulation of the invention when fed to animals provides a means to decrease populations of undesirable gastrointestinal microbes and results in increased weight gain of the animals, especially under the less than optimum growth conditions normally present in commercial livestock environments.

Other aspects and features of the invention will be more fully apparent from the following disclosure and

appended claims.

DETAILED DESCRIPTION OF THE INVENTION AND PREFERRED EMBODIMENTS THEREOF

The present invention provides a formulation usable as a food or feed additive for animals. In the broadest aspect of the invention, animals may be fed the additive in a variety of ways: for example, (1) the additive may be combined with dry feed during feed milling or when the feed is delivered to the animals; (2) the additive may be sprinkled on the food as a powder; or (3) the additive may be mixed in the drinking water. Preferably, to minimize labor, the additive is mixed with dry feed.

In the particular invention, such animals specifically include all poultry and mammals, including human beings. In its most basic form, the formulation for a particular animal comprises one or more pure cultures of a Lactobacillus species naturally occurring in the gastrointestinal tract of that animal and a source of a sugar that is metabolizable by the Lactobacillus species but not to any great extent by the animal. Thus, the formulation of the invention comprises:

(a) a bacterial culture comprising at least one live pure culture of a Lactobacillus species which occurs naturally in a particular animal group; and

(b) a source of sugar metabolizable by the Lactobacillus species in the bacterial culture but not metabolizable by the animals in the group.

By the term "group" is meant animals of a particular species or group of species which share in common a tendency to have a similar gastrointestinal Lactobacillus flora and a similar inability to metabolize a sugar which is metabolizable by the Lactobacillus flora. As discussed below, the formulation discussed in detail herein has been

devised for poultry but is adaptable to other animals, and includes a source of lactose which is not metabolizable by poultry.

In the preferred embodiment, the formulation comprises a live, pure culture of at least one of Lactobacillus reuteri, L. animalis and L. salivarius, and a sugar source.

The preferred sugar source is whey, because it is inexpensive and easily available, and because it contains lactose, a good source of carbon and energy for growth of the added microorganisms. An additional advantage of using a lactose source for feeding poultry or other birds is that birds do not utilize this sugar, and it is therefore readily available for the added microorganisms. Preferably, powdered whey is utilized as the lactose source to minimize shipping costs and spoilage prior to formulation of the additive.

The preferred method of formulating the additive is as follows. L.reuteri, L.animalis and/or L.salivarius are grown individually in a variety of appropriate media used for lactobacilli. Lactose or maltose are the preferred sources of energy so that the cells are capable of rapid metabolism of the carbohydrates which may be present in the formulation or in the animals' food. The cells used for the preparation of the additive may be freshly harvested, frozen, lyophilized or suspended in oil or a specifically formulated diluent such as an aqueous solution. Commercially available whey powder or whey concentrate is used to formulate the additive. Although the cells and whey may be fed separately, the are preferably mixed together with or without other ingredients (e.g. corn, soybean meal, wheat, etc.). The mixture may be of a variety of microbe and whey mixtures, for example a solid and a solid (e.g. fine powder with granulated whey, etc.), a liquid and a solid (cell suspension and whey), a solid and a liquid (lyophilized cells and a liquid whey concentrate) or a liquid and a liquid (liquid cell

suspension and a liquid whey concentrate). The additive final presentation of the mixture could be as a powder, granules, or pellets or liquid.

In its most preferable form, the invention comprises: a method of delivering DFM's to birds so that the DFM's are established in the gastrointestinal tract. L. reuteri cells or other DFM's are incorporated onto the surface or within pellets. The pellets may be fed to the birds, for example poultry, along with the birds' normal diet.

Lyophilized (freeze-dried) Lactobacilli reuteri strains, T-1 (isolated from turkey) and 11284 (isolated from chickens), when held at room temperature (approximately 25°C) are found to remain viable for as long as 30 days but to decrease in number. For example, a population of 6×10^6 colony forming units (CFU)/g were recovered of the original 3×10^{10} CFU/g at 30 days. It was found that when the lyophilized cells were suspended in an oil, such as sunflower oil at room temperature for 30 days, no loss of viability was observed.

The invention provides in its one preferred embodiment that lyophilized L. reuteri cells suspended in oil are coated over pelletized whey particles. Under room temperature, no decrease in viability is observed for up to seven days. When the Lactobacillus coated pelletized particles of whey are mixed with poultry feed, no significant loss of viability occurs over four days at room temperature.

In another preferred embodiment of the invention Lactobacillus reuteri cells in oil are mixed with whey powder and then the mixture is compressed into pellets or tablets. Although survival is lower than in the first embodiment when there is no cooling in the pelletization process, survival is sufficient for use of the pellets as a beneficial food additive which aids in establishing the DFM in the animal.

The features and advantages of the present invention

will be more clearly understood by reference to the following examples, which are not to be construed as limiting the invention.

Example 1. Growth of Turkey Poults to be Fed Additive

One day old Nicholas turkey tom poults are used in this study. The poults are not toe clipped, desnooded or wing clipped, nor are they given any vaccinations.

The turkeys are placed in animal rooms at the Dearstyne Poultry Research Center, Department of Poultry Science at NCSU's Agricultural Research Service (NCARS). The animal rooms have controlled ambient temperature, day length and thermostatically controlled Petersime brooding batteries (Petersime Incubator Co., Petersime, OH).

A normal turkey starter diet, for example as shown in Table 1 with and without whey powder, is used throughout the trial. The amount of whey in the diet allows for a final 2.2% lactose. The trial is twenty days in duration, covering the period from day of hatch to day 12. The turkeys are weighed on Day 0 (at hatch), Day 5, Day 12, Day 15 and Day 20.

Table 1.

INGREDIENT	TURKEY STARTER DIET CONTROL (lbs/1000)	PLUS WHEY (lbs/1000)
Soy	490	487
Corn	405	373
Whey (73% lactose)	---	30
Poultry Fat	44.4	49.6
Ethoxyquin	0.12	0.12
Dicalcium phosphate	35.5	35.5
Limestone	14.5	14.5
Sodium Chloride	3.7	3.7
Trace minerals	1.0	1.0
Vitamin mix,	1.0	1.0
Choline Cl (60%)	2.0	2.0
L-Lys.HCl	0.1	0.1
DL-Met	2.8	2.8
	1000.0	1000.0

Example 2. Growth and Quantitation of Bacterial Cultures

L.reuteri 11284, known to colonize the chicken GI tract, and L.reuteri T1, which is a strain isolated from turkeys, are the strains which are used. The Lactobacillus strains are grown in LCM medium utilizing lactose or maltose for 24 h at 37°C, harvested by centrifugation, and washed twice with fresh basal medium as previously described (2, 6). These cells are mixed into the animal feed at a level of approximately 10^5 CFU g^{-1} of feed. This inoculum level has been shown to effectively enhance the population level of this microorganism in the chicken ceca (Casas et al., 1990, in preparation). The number of L. reuteri in the feed and in the ceca are

monitored as previously described (6). Appropriate dilutions are plated onto LBS agar and incubated anaerobically (Gas-Pak jars) at 37°C for 48 h. Plates containing about 50 to 200 CFU are overlaid with glycerol agar seeded with L. plantarum indicator cells, reincubated anaerobically for 24 h, and colonies showing growth inhibition zones counted as reuterin-producing L.reuteri cells.

S. senftenberg, isolated from turkeys, resistant to novobiocin and nalidixic acid, was obtained from Evillmar Poultry Co. (Evillmar, MN). Inocula for infectious challenge are prepared from cultures grown in BHI Broth (Difco Laboratories, Inc., Detroit, MI) and incubated at 37°C for 24 hours. The cultures are diluted appropriately in sterile 50 mM phosphate buffer, pH 7.0, to obtain challenge inocula containing 10^6 CFU per ml. Enumeration of these Salmonella is carried out by plating appropriate dilutions on Salmonella medium (35).

Caecal content samples for microbiological enumeration are prepared from sacrificed birds. Caeca are carefully removed from the birds and the open end of each is clipped. The exterior of the caecum is alcohol sterilized before transferring its contents to a stomacher bag for mixing and further dilution.

Example 3. Treatment of Poults

Turkey poults of Example 1 are subjected to the following eight treatments with two pens of 15 birds per pen being in each group:

Salmonella senftenberg infected group

1. Control, no whey, no L.reuteri
2. No whey, L.reuteri
3. Whey, no L.reuteri
4. Whey, L.reuteri

L.reuteri, when administered is mixed into the feed. The inoculated feed is changed every two days to guarantee

the presence of viable L. reuteri in the feed. Whey is added to the feed, before milling, for a final 5% lactose concentration. Alternatively, L. reuteri and concentrated or dehydrated whey are formed into tablets , or added together in any product in which both components in a liquid or solid form have been previously combined, and the combination added to the feed of the animals.

S. senftenberg (10^6 CFU per ml) is crop fed by the means of an animal feeding stainless steel needle attached to a hypodermic syringe on day 5 after hatch.

Example 4. Results of Adding Lactobacillus, Whey and Salmonella

Salmonella senftenberg in feces and caecal contents of poults treated as in Example 3 is shown in Tables 2 and 3, respectively. The effect of L. reuteri and whey on the number of S. senftenberg in feces (droppings) becomes obvious at 72 h after Salmonella challenge. The data indicate a synergistic effect when whey and L. reuteri are added together.

The presence of S. senftenberg in caecal contents is presented in Table 3. The results show that addition of L. reuteri and/or whey, but in particular, the combination of L. reuteri and whey, is effective in reducing the presence of S. senftenberg in the ceca of these animals. Thus, whereas 47% of the control ceca tested positive for S. senftenberg, none (0%) of the samples tested positive when fed L. reuteri and whey.

TABLE 2. Log_{10} CFU of S. senftenberg per g feces 72 h post challenge.

	TREATMENTS			
REPLICA SAMPLES	CONTROL	L.reuteri	WHEY	WHEY AND L.reuteri
a.	> 10	6	3	4
b.	9	7	8	5
c.	> 10	7	6	< 3
d.	10	3	< 3	< 3

TABLE 3. Percent of cecal samples testing positive for S. senftenberg and L. reuteri 7 days post challenge

TREATMENTS	L.reuteri (%)[1]	S.senftenberg (%)[2]
CONTROL	0	47
L.reuteri	29	40
WHEY	6	13
WHEY AND L.reuteri	82	0

[1] Positive samples had > 10^7 CFU/g
[2] Positive samples had < 10^3 CFU/g

Example 5. Growth of Cold Stressed Poults Fed with L. reuteri and Whey

Instead of exposing turkey poults to constant temperature rooms as in Example 1, the temperature in the pens of cold stressed birds is 90 degrees F for 1 hour, then 85 degrees G for 2 hours in an on-off cycling for 48 hours after hatch. The temperature is then set back to normal brooding temperature for the remainder of the

experiment, normal brooding temperature being 90 degrees F for the first seven days after hatch, 85 degrees F from day 7 to day 10, and 75 to 80 degrees after day 10.

Turkey poults are subjected to the following four treatments, with eight pens of eight birds per pen being in each treatment group.

1. Control, no whey, no L. reuteri
2. No whey, L. reuteri
3. Whey, no L. reuteri
4. Whey, L. reuteri

Example 6. Results of Growth of Cold Stressed Poults

Relative wights of poults treated as in Example 5, at 0, 5, 10, 15, and 20 days of age are shown in Table 4. The beneficial effect of whey becomes evident at day 5, while the effect of L. reuteri becomes obvious at days 15 and 20.

TABLE 4. Effect of L. reuteri and whey on body weight of turkey poults.

TREATMENTS	RELATIVE WEIGHT (PERCENT) AT				
	Day 0	Day 5	Day 10	Day 15	Day 20
No whey, no L.reuteri	100	100	100	100	100
No whey, plus L. reuteri	99	98	102	104	105
Plus whey, no L. reuteri	99	103	101	101	103
Plus whey, plus L. reuteri	99	103	103	100	105

For percentage conversion, control weights (no whey, no L. reuteri) at each weight day were made equal to 100%.

Example 7. Use of Lactobacillus salivarius and Lactobacillus animalis

L. salivarius subsp. salivarius ATCC type strain No. 11741 and L. animalis ATCC type strain No. 35046 are grown

as in Example 2. Each strain is added individually to feed as in Example 3. The feed is augmented with whey according to Example 3. The feed is used to feed chickens and turkeys to decrease undesirable microbial organisms and improve poultry weight gain.

Example 8. Use of Multiple Lactobacillus Strains

Strains of the three Lactobacillus species discussed in Examples 3 and 8 are each added individually, or as a mixed inoculum to the whey-augmented feed according to Example 3. The feed containing the three strains is used to feed turkeys and chickens.

Example 9. Formation of Pellets

Powdered whey is exposed to compaction at a pressure of 10-15 lb/in^2 to form pellets. The pellets are milled and sieved to a size which is edible by the birds, for example, -8, +20 mesh for little pellets and -1/4", +8 mesh for larger pellets. Lactobacillus reuteri strain T-1, 11284 or other strains compatible with the intended host animal species are lyophilized in a cryoprotectant such as milk or whey and then is mixed in an oil, such as a sunflower oil-based drench at a concentration of about 3×10^{10}/g in the oil. The drench may contain trace amounts of silicon dioxide.

The strains mentioned above have been deposited at the American Type Culture Collection in Rockville, Maryland.

The pellets of whey are then coated with the Lactobacillus-containing oil which may be done simply by pouring the oil-suspension over the whey pellets so that there are about 5×10^7 to about 10^8 cells/g whey. The survival of the Lactobacillus on the pellets is shown in the first column of data in Table 5. The whey particles are then mixed with feed pellets or particles so that the whey particles comprise 2 - 5% of the feed by weight, so that there are 5×10^5 to 10^6 CFU/g feed mix.

Table 5

Time (days)	Oil drench	In Product	Product feed
0	3×10^{10}	6×10^{8}	7×10^{6}
1	ND	3×10^{8}	9×10^{6}
2	ND	2×10^{8}	4×10^{6}
3	4×10^{10}	6×10^{8}	7×10^{6}
4	ND	ND	4×10^{6}
5	ND	3×10^{8}	ND
7	3×10^{10}	3×10^{8}	ND
10	4×10^{10}	ND	ND
20	3×10^{10}	ND	ND
30	3×10^{10}	ND	ND

Example 10. Survival of Lactobacillus in Pellets

A Lactobacillus-oil suspension is prepared as in Example 9. The suspension is then mixed with whey powder in a concentration of 10^7 per g whey. The mixture is then compacted, milled and sieved as in Example 9. Typical results of survival of the Lactobacillus reuteri in such pellets is shown in the central data column of Table 5. The survival when such pellets are mixed with feed as done in Example 9 is shown in the final column of Table 5.

Example 11. Turkeys Fed Pellets

Turkey poults are fed feed and pellets having about 10^7 CFU L. reuteri/g feed prepared according to Example 10 for a period of 10 days. The total number of lactobacilli found in the bird's cecum is determined for each treatment as colony-forming units per excised and homogenized cecum. Solid Lactobacillus selection medium (1.5% agar) as described in references 2, 5, and 7 is used. The percent of the colonies which were L. reuteri is determined as described in international patent application PCT/US88/01423 but using L. plantarum as the indicator organism. In this test, colonies of lactobacilli on the LBS agar medium are overlaid with 10 ml of 1% liquified

agar containing 0.5 M glycerol and a L. plantarum inoculum. After anaerobic (GAS-Pack System) incubation at 37°C for 24 hours, zones of growth inhibition are seen around colonies that produce reuterin from glycerol. These colonies are thus identified and enumerated as L. reuteri.

As seen in Table 6, colonization of the ceca by L. reuteri is enhanced by the feed treatment as compared to the control. Only 1/5 of the control birds in the results shown are positive for L. reuteri, while 4/5 of the treated birds retain significant numbers of L. reuteri in the cecum.

Table 6

	CFU per g Ceca		% Of Birds Positive For L. reuteri
	Total Lactobacilli	L. reuteri	
Control birds	9.0×10^8 to 1.5×10^{10}	1.5×10^5 to 1.2×10^8	20%
Treated birds	5.0×10^7 to 3.7×10^9	4.0×10^7 to 1.1×10^9	80%

While the invention has been described with reference to specific embodiments thereof, it will be appreciated that numerous variations, modifications, and embodiments are possible, and accordingly all such variations, modifications, and embodiments are to be regarded as being within the spirit and scope of the invention.

BEST MODE FOR CARRYING OUT THE INVENTION

A formulated product that may be used as an animal feed additive includes isolated and identified pure culture(s) of naturally occurring gastrointestinal microorganisms. Preferably viable cells of Lactobacillus reuteri, an oil and whey powder are used. The Lactobacillus cells may be coated on the surface of whey

pellets or be contained in the pellets. Preferably powdered whey is exposed to compaction to form pellets. The pellets are milled and sieved to a size which is edible by the birds.

INDUSTRIAL APPLICABILITY

The invention includes a formulated product that may be used as an animal feed additive. The feed additive provides Lactobacillus cells to the animal, resulting in decreased survival of gastrointestinal pathogens and increased animal weight gain.

REFERENCES

1. Axelsson L, Lindgren SE. 1987. Characterization and DNA homology of Lactobacillus reuteri strains isolated from pig intestine. J. Appl. Bacteriol. 62:433-440.

2. Axelsson L, Chung TC, Dobrogosz WJ, Lindgren SE. 1989. Production of a broad spectrum antimicrobial substance by Lactobacillus reuteri. Microbial Ecol. Health Dis. 2:131-136.

3. Bailey JS. 1988. Integrated colonization control of Salmonella in poultry. Poultry Sci. 67:928-932.

4. Bechman TL, Chambers JV, Cunningham MD. 1977. Influence of Lactobacillus acidophilus on performance of young diary calves. J. Dairy Sci. 60:74(abs).

5. Bruce BB, Gilliland SE, Bush LJ, Staley TE. 1979. Influence of feeding cells of Lactobacillus acidophilus on the fecal flora of young calves. Oklahoma Anim. Sci. Res. Rep. 207.

6. Chung TC, Axelsson L, Lindgren Se, Dobrogosz WJ. 1989. In vitro studies on reuterin synthesis by Lactobacillus reuteri. Microbial Ecol. Health Dis. 2:137-144.

7. Corrier D, Hinton, Jr. A, Ziprin RL, Beier RC, DeLoach JR. 1990. Avian Dis. 34:617-625.

21. Corrier D, Hinton, Jr. A, Ziprin RL, DeLoach JR. 1990. Avian Dis. 34:668-676.

8. Damron BL, Wilson HR, Voitle RA, Harms RH. 1981. A mixed Lactobacillus culture in the diet of broad breasted large white turkey hens. Poultry Sci. 60:1350-1351.

9. Dellaglio F, Arrizza FS, Leda A. 1981. Classification of citrate fermenting lactobacilli isolated from lamb stomach, sheep milk and pecorino romano cheese. Zbl. Bakt. Hyg., Abt. Orig. C2:349-356.

10. Dilworth BC, Day EJ. 1978. Lactobacillus cultures in broiler diets. Poultry Sci. 57:1101.

11. Dobrogosz WJ, Casas IA, Pagano GA, Talarico TL, Sjorberg B-M, Karlson M. 1989. Lactobacillus reuteri and

the enteric microbiota. In: The Regulatory and Protective Role of the Normal Microflora (Eds: Grubb R, Midtvedt T, Norin E) Macmillan LTD, London, pp. 283-292.

12. Ellinger DK, Muller LD, Gantz PJ. 1978. Influence of feeding fermented colostrum and Lactobacillus acidophilus on fecal flora and selected blood parameters of young dairy calves. J. Dairy Sci. 61:162(abs).

13. Food and Drug Administration Compliance Policy Guide No. 7126.41, May 2, 1988.

14. Fox SM. 1988. Probiotics: Intestinal inoculants for production animals. Food-Animal Practice, Vet. Med., August issue.

15. Francis C, Janky DM, Arafa AS, Harms RH. 1978. Interrelationship of Lactobacillus and zinc bacitracin in the diets of turkey poults. Poultry Sci. 57:1687-1689.

16. Fuller R. 1986. Probiotics. J. Appl. Bacteriol. Symp. Suppl. 1S-7S.

17. Goodenough ER, Kleyn DH. 1976. Influence of viable yoghurt microflora on the digestion of lactose by the rat. J. Dairy Sci. 59:601-606.

18. Goodling AC, Cerniglia, GJ, Herbert JA. 1987. Production performance of white leghorn layers fed Lactobacillus fermentation products. Poultry Sci. 66:480-485.

19. Hargis P, Creger CR. 1978. Lactobacillus fermentation product in laying hen rations. Poultry Sci. 57:1103.

20. Hatch RC, Thomas RO, Thayne WV. 1973. Effect of adding Bacillus acidophilus to milk fed to baby calves. J. Dairy Sci. 56:682(abs).

22. Impey CS, Mead GC, George SM. 1982. Competitive exclusion of salmonellas from the chick caecum using a defined mixture of bacterial isolates from the caecal microflora of an adult bird. J. Hyg. Camb. 89:749.

23. Kandler O, Stetter K, Kohl R. 1980. Lactobacillus reuteri sp. nov. a new species of heterofermentative lactobacilli. Zbl. Bakt. Hyg. Abt. Orig. C1:264-269.

24. Kandler O, Weiss N, 1986. Regular nonsporing Gram positive rods. Bergey's Manual of Systematic Bacteriology (Eds.: Sneath DHA, Mair NC, Sharpe ME, Holt JH), vol. 2:1208-1234. Williams and Wilkins, NY.

25. Mead GC, Impey CS. 1986. Current progress in reducing salmonella colonization of poultry by 'competitive' exclusion. J. Bacteriol. Symp. Suppl. 675-755.

26. Metchnikoff E. 1907. Prolongation of Life. Heinemann, London.

27. Morrill JL, Dayton AD, Mickelson R. 1977. Cultured milks and antibiotics for young calves. J. Dairy Sci. 60:1105.

28. Muralidhara KS, Sheggeby GG, Elliker PR, England DC, Sandine WE. 1977. Effects of feeding lactobacilli on the coliform and Lactobacillus flora of intestine tissue and feces from piglets. J. Food Protection 40:288-295.

29. Nurmi E, Rantala M. 1973. New aspects of Salmonella infection in broiler production. Nature 241:210-211.

30. Orla-Jensen S. 1943. The lactic acid bacteria. Det Kongelige Danske Videnskasbernes Selskab. Biologiske Skrifter, Bind II, Nr. 3. Kobenhavn.

31. Oyofo BA, DeLoach JR, Corrier, DE, Norman JO, Ziprin RL, Mollenhauer HH. 1989. Effect of carbohydrates on Salmonella typhimurium colonization in broiler chickens. Avian Dis. 33:531-534.

32. Parker RB. 1974. Probiotics, the other half of the antibiotic story. Anim. Nutr. Health. 29:4-8.

33. Parkhurst CR, Edens FW, Casas IA. 1991. Lactobacillus reuteri and whey reduce Salmonella colonization in turkey poults. International Poultry Trade Show, Southeastern Poultry and Egg Association, Atlanta, GA, Abs. Sci. Meet., Jan. 30 - Feb. 1, 1991.

34. Potter LM, Bliss BA, Blair ME. 1986. Lactobacillus acidophilus compared to bacitracin as a growth promotant for young turkeys. Poultry Sci. (Suppl. 1) 65:107.

35. Rembach. 1990. Appl. Environ. Microbiol. 56:301-303.

36. REVUE: Scientifique et Technique, Digestive Microflora and Bioregulation, International Office Of Epizootics, F-75017, Paris, France, Vol., 8, June, 1989.

37. Sarra PG, Magri M, Bottazzi V, Dellaglio F, Bosi E. 1979. Frequenza di bacilli heterofementanti nelle feci di vitelli lattanti. Arch. Vet. Ital. 30-16-21.

38. Sarra PG, Dellaglio F, Bottazzi V. 1985. Taxonomy of lactobacilli isolated from the alimentary tract of chickens. System. Appl. Microbiol. 6:86-89.

39. Sarra PG, Vescovo M, Fulgoni M. 1986. Study on crop adhesion genetic determinant in Lactobacillus reuteri. Microbiologica 9:279-285.

40. Sissons JW. 1989. Potential of probiotic organisms to prevent diarrhoea and promote digestion in farm animals--a review. J. Sci. Food Agric. 46:1-13.

41. Snoeyenbos GH, Weinack OM, Smyser CF. 1978. Protecting chicks and poults by oral administration of "normal" gut microflora. Avian Dis. 22:273-287.

42. Soerjadi AS, Stehman SM, Snoeyenbos GH, Winack OM, Smyser CF. 1981. The influence of lactobacilli on competitive exclusion of paratyphoid salmonellae in chickens. Avian Dis. 25:1027-1033.

43. Speck ML. 1977. Heated yoghurt--is it still yoghurt? J. Food Protection. 40:863-865.

44. Talarico TL, Casas IA, Chung TC, Dobrogosz WJ. 1988. Production and isolation of reuterin: a growth inhibitor produced by Lactobacillus reuteri. Antimicrob. Agents. Chemotherap. 32:1854-1858.

45. Talarico TL, Dobrogosz WJ. 1989. Chemical characterization of an antimicrobial substance produced by Lactobacillus reuteri. Antimicrob. Agents Chemotherap. 33:674-679.

46. Talarico TL, Dobrogosz WJ. 1990. Purification and characterization of glycerol dehydratase from Lactobacillus reuteri. Appl. Environ. Microbiol. 56:1195-1197.

47. Talarico Tl, Axelsson L, Novotny J, Fiuzat M,

Dobrogosz WJ. 1990. Utilization of glycerol as a hydrogen acceptor by Lactobacillus reuteri: Purification of 1,3-propanediol:NAD oxidoreductase. Appl. Environ. Microbiol. 56:943-948.

48. Tortuero F. 1973. Influence of the implantation of Lactobacillus acidophilus in chicks on the growth, feed conversion, malabsorption of fats syndrome and intestinal flora. Poultry Sci. 52:197-203.

49. Underdahl NR, Torres-Medina A, Doster AR. 1982. Effect of Streptococcus faecium C-68 in control of Escherichia coli- induced diarrhoea in gnotobiotic pigs. Amer. J. Vet. Res. 43:2227-2232.

50. Vescovo M, Morelli L, Cocconcelli PS, Bottazzi V. 1984. Protoplast formation, regeneration, and plasmid curing in Lactobacillus reuteri. FEMS Microbiol. Lett. 23:333-334.

51. Watkins BA, Kratzer FH. 1982. Effects of varying dose levels of Lactobacillus strains on gut colonization and chick performance. Poultry Sci. 61:1565.

52. Watkins BA, Kratzer FH. 1984. Drinking water treatment with a commercial preparation of a concentrated Lactobacillus culture for broiler chickens. Poultry Sci. 63:1671-1673.

53. Watkins BA, Miller BF. 1983. Competitive gut exclusion of avian pathogens by Lactobacillus acidophilus by gnotobiotic chicks. Poultry Sci. 62:1772-1779.

54. Wierup M, Wold-troell M. 1988. Epidemiological evaluation of the Salmonella-Controlling effect of a nationwide use of a competitive exclusion culture in poultry. Poultry Sci. 67:1026.-1033

THE CLAIMS

What Is Claimed Is:

1. A method of establishing a direct feed microorganism in the gastrointestinal tract of an avian organism, comprising adding lyophilized direct fed microorganisms to a whey pellet.

2. A method according to claim 1 wherein the microorganism is Lactobacillus reuteri.

3. A method according to claim 2 wherein the avian organism is a chicken.

4. A method according to claim 2 wherein the avian organism is a turkey.

5. A method according to claim 1, comprising coating the outside of whey pellets with direct feed microorganisms in an oil suspension.

6. A method according to claim 1, comprising mixing a direct feed microorganism in an oil suspension with whey powder, and compacting the mixture into pellets.

7. A feed additive, comprising a pellet comprising a pure culture of direct feed microorganism and whey.

8. A feed additive according to claim 7, wherein the direct feed microorganism comprises lyophilized Lactobacillus reuteri cells.

9. A feed additive according to claim 8, wherein the L.reuteri cells are in an oil suspension.

10. A feed additive according to claim 9, wherein the suspension of L. reuteri cells is coated on the

outside surface of whey pellets.

11. A feed additive according to claim 9, wherein the suspension of L. reuteri cells is mixed with whey powder and compacted into a pellet.

12. A formulation for oral administration to poultry, comprising:

(a) a bacterial culture comprising at least one live pure culture of a Lactobacillus species which occurs naturally in gastrointestinal tracts of poultry, said Lactobacillus species selected from the group consisting of L. reuteri, L. salivarius, and L. animalis; and

(b) a source of sugar metabolizable by the Lactobacillus species in the bacterial culture but not metabolizable by poultry, wherein said formulation is capable of reducing Salmonella in poultry and provides a concentration of about 2% to about 5% of said sugar in poultry feed when the formulation has been added to said feed to provide a level of Lactobacillus sufficient to reduce Salmonella in the gastrointestinal tract of poultry, and wherein said formulation when fed to poultry results in greater numbers of Lactobacillus cells in the gastrointestinal tract than when poultry is fed Lactobacillus cells without the sugar.

13. A formulation for oral administration according to claim 12, wherein the source of sugar is lactose.

14. A formulation for oral administration according to Claim 12, wherein the source of sugar comprises whey.

15. A formulation for oral administration according to

claim 13, wherein the lactose is in whey.

16. A method of decreasing numbers of undesirable microbes in an animal's gastrointestinal tract, comprising:

(a) obtaining at least one pure culture of Lactobacillus cells;

(b) obtaining a source of sugar; and

(c) administering the Lactobacillus cells and the sugar orally to the animal.

17. A method of decreasing numbers of undesirable microbes in gastrointestinal tracts of animals, comprising:

(a) obtaining at least one pure culture of Lactobacillus cells;

(b) obtaining a source of sugar metabolizable by the Lactobacillus cells and not to a significant extent by the animals or the undesirable microbes; and

(c) administering the Lactobacillus cells and the sugar orally to the animal.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/00708

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): A01N 63/00; A23L 1/00; C12N 1/20
US CL : 424/93; 426/2; 435/252.9, 853

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	424/93; 426/2; 435/252.9, 853

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
Y	US, A, 3,984,575 (Farr) 05 October 1976, see entire document.	1-17
Y	US, A, 4,335,107 (Snoeyenbos et al) 15 June 1982, see entire document.	1-17
Y	US, A, 4,518,696 (Gehrman et al) 21 May 1985, see entire document.	1-17
Y	Avian Diseases, Vol. 22, No. 2, issued 1978, Snoeyenbos et al, "Protecting Chicks and Poults from Salmonellae by Oral Aministration of 'Normal' Gut Microflora", pages 273-287, see entire document.	1-17
Y	Avian Diseases, Vol. 33, issued 1989, Oyofoet al, "Effect of Carbohydrates on Salmonella typhimurium Colonization in Broiler Chickens", pages 531-534, see entire document.	1-17
Y	Microbial Ecology in Health and Disease, Vol. 2. issued 1989, Chung et al, "In Vitro Studies on Reuterin Synthesis by Lactobacillus Reuteri", pages 137-144, see entire document.	1-17

* Special categories of cited documents:[15]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
30 MARCH 1992	10 JUN 1992
International Searching Authority[1]	**Signature of Authorized Officer[20]**
ISA/US	SUSAN M. WEBER

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